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Glycoside Hydrolase Activities of Thermophilic Bacterial Consortia Adapted to Switchgrass

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Abstract

Industrial scale biofuel production requires robust enzymatic cocktails to produce fermentable sugars from lignocellulosic biomass. Thermophilic bacterial consortia are a potential source of cellulases and hemicellulases adapted to harsher reaction conditions than commercial fungal enzymes. Compost-derived microbial consortia were adapted to switchgrass at 60°C to develop thermophilic biomass-degrading consortia for detailed studies. Microbial community analysis using SSU rRNA gene amplicon pyrosequencing and short-read metagenomic sequencing demonstrated that thermophilic adaptation to switchgrass resulted in low diversity bacterial consortia with a high abundance of bacteria related to thermophilic *Paenibacilli*, *Rhodothermus marinus*, and *Thermus thermophilus.* At lower abundance, thermophilic *Chloroflexi* and an uncultivated lineage of the *Gemmatimonadetes* phylum were observed. Supernatants isolated from these consortia had high levels of xylanase and endoglucanase activity. Compared to commercial enzyme preparations, the endoglucanase enzymes had a higher thermotolerance and were more stable in the presence of 1-ethyl-3-methylimidazolium acetate ([C2mim][OAc]), an ionic liquid used for biomass pretreatment. The supernatants were used to saccharify [C2mim][OAc]-pretreated switchgrass at elevated temperatures (up to 80°C), demonstrating that these consortia are an excellent source of enzymes for the development of enzymatic cocktails tailored to more extreme reaction conditions.

Introduction

Enzyme cocktails that hydrolyze plant cell wall polysaccharides are a critical component of bioprocessing configurations designed to transform lignocellulosic biomass into biofuels (12, 24). The large variety of potential biomass feedstocks and pretreatments available require tailored glycoside hydrolase cocktails that function optimally under diverse conditions, including high temperatures, extreme pH, and the presence of residual pretreatment chemicals and inhibitors (13, 25). Current commercial cocktails consist of preparations of fungal-derived glycoside hydrolases, primarily cellulases and hemicellulases (3, 18). However, fungal enzymes are often deactivated by elevated temperatures or by residual chemicals from pretreatment (3). For example, ionic liquids (ILs) such as 1-ethyl-3-methylimidazolium acetate ([C2mim][OAc]) can dissolve lignocellulosic biomass and dramatically improve cellulose hydrolysis kinetics (11, 22, 28), yet multiple studies have shown that fungal endoglucanases are deactivated at low levels of ILs that may persist in the biomass after pretreatment (4, 33). In contrast, thermophilic bacterial and archaeal endoglucanases have been shown to be active in >20% [C2mim][OAc], suggesting that thermophilic prokaryotes may be an important source of enzymes for the development of more robust enzyme cocktails (5).

Aerobic, thermophilic bacteria are a potentially rich source of glycoside hydrolases for biomass deconstruction. However, these bacteria generally secrete low levels of glycoside hydrolases, especially cellulolytic enzymes (24). Recent efforts to identify these enzymes have involved functional screening of expression libraries or bioinformatic homology-based searches of sequences derived from isolated organisms or environmental

samples (1, 35). These approaches have significant limitations: expression libraries may miss relevant genes due to low representation within the library and poor expression in laboratory host strains, while bioinformatic approaches suffer mainly from the limited ability to predict the specific characteristics of an enzyme (activity, thermostability, etc.), which is often based on homology to known enzymes (29). The complexity of microbial communities in environmental samples also hampers bioinformatic enzyme discovery efforts because it often prevents assembly of full-length genes from metagenomic databases (1, 6). Enrichment cultures on lignocellulosic biomass provide a method to reduce the complexity of the microbial communities and provide more tractable samples for detailed investigations. In previous studies, simplified bacterial communities have been cultivated at elevated temperature with cellulose or biomass as substrates (23, 27). These studies have focused on the isolation of thermophilic bacteria from the enrichments and the characterization of glycoside hydrolases secreted by these isolates. Here, we describe an alternative approach to discovering robust and highly active lignocellulosic-biomass degrading glycoside hydrolases, in which enrichments are performed using a targeted biomass feedstock, switchgrass, and the enriched microbial consortia are studied directly using culture-independent approaches and glycoside hydrolase assays.

Materials and Methods

Environmental Samples

The compost inocula for switchgrass-adapted cultures were collected from two municipal green waste composting facilities. The green waste consisted of yard trimmings and discarded food waste. The first facility was Grover Soil Solutions located in Zamora, CA. Samples collected from this site are refered to as Zamora (Z) and were collected as previously described (1). The second facility was Jepson Prairie Organics located in Vacaville, CA. Samples collected from this site are referred to as Jepson Prairie (JP). This facility composts municipal green waste in watered and turned windrows. Compost was collected from windrows in the mesophilic (7 days) and thermophilic (30 and 60 day) composting stages. A spade was used to remove the top 12 inches of each windrow and the exposed biomass was placed into 50 mL Falcon tubes, transported at room temperature, and frozen at -80°C within 2 h.

Cultivation Conditions

Compost microbial communities were adapted to ground switchgrass (*Panicum virgatum* L.) as their sole carbon source by serially passing the community through nine liquid cultures (Table 1). Chemical characterization of the switchgrass cultivar has been previously described (22). The switchgrass was exhaustively extracted with water and ethanol in a Soxhlet apparatus to remove soluble sugars and other nutrients, then dried at 50°C prior to use. Detailed procedures for the enrichments are described in Supplemental Methods.

Isolation of Culture Supernatants

The final cultures (passage #9) were used for glycoside hydrolase assays and all passages were prepared for DNA isolation. The culture supernatant was clarified by decanting 30 ml of the culture supernatant into several 2 ml centrifuge tubes, and spinning at 21,000 x *g* for 10 min. The supernatant was removed and the pellets from four tubes consisting of the switchgrass-adapted microbial community and particles of switchgrass were combined, transferred into 2 ml Lysing Matrix E tubes (Qbiogene**,** Montreal, QN), and frozen at -80°C for DNA extraction. The clarified supernatants were pooled in 50 mL Falcon tubes and passed through a 0.2 µm filter; this supernatant was used directly for measuring glycoside hydrolase activity. For zymography, contaminating lignin-derived phenolic compounds were removed from the clarified supernatant by adding polyethyleneimine to a final concentration of 0.1% to 1 ml of supernatant, shaking for 2 h at 4°C, and centrifuging at 10,000 *x g* for 20 min at 4°C.

SSU rRNA Amplicon Pyrosequencing

DNA was isolated from the pellets generated during isolation of culture supernatants described above. DNA isolation and sequencing were performed as previously described (6). Sequencing tags were quality trimmed and analyzed using the pyroclust version of the software tool PyroTagger (http://pyrotagger.jgi-psf.org) with a 220 bp sequence length threshold and an accuracy of 10% for low quality bases (10, 20). All singleton OTUs were removed from the data set to reduce noise in statistical analysis. The raw

pyrotags reads are available in the NCBI Short Read Archive (SRA030499, SRA030513, SRA030539).

Illumina Sequencing and EMIRGE Reconstruction of SSU rRNA Genes

Illumina libraries were constructed and sequenced from DNA isolated from Z-9 1%SG and JP-9 1%SG using previously described protocols (26). Libraries of paired-end 76bp reads were created for JP-9 1% SG (4.8 GB) and Z-9 1% SG (1.3 GB). Near full length ribosomal small subunit genes were reconstructed from Illumina sequencing reads using EMIRGE (26). For both communities, all reads were trimmed from the 3' end until a base with quality score ≥ 3 was encountered. Paired-end reads where both reads were at least 60 nucleotides in length after trimming were used as inputs to EMIRGE, with the reference database as previously described (26). Data from each community was processed separately for 80 iterations, and SSU sequences, 23 in total, with relative abundances > 1% were kept for further analysis. Chimera check with Bellepheron (greengenes.lbl.gov) and manual analysis of EMIRGE-derived sequences excluded two of these sequences at 1.1% and 2.7% abundance, respectively. The raw metagenomic reads are available through the Integrated Microbial Genomes with Microbiome Samples website maintained by the Joint Genome Institute, Walnut Creek, CA (http://img.jgi.doe.gov/cgi-bin/m/main.cgi).

Phylogenetic Tree Construction

Maximum Likelihood trees were built with RAxML (31), using the GTRGAMMA model of nucleotide substitution and 100 bootstrapped replicates. Sequences were first aligned

with Muscle (9) using default parameters, and columns in the full alignment with gaps were removed from the alignment for tree construction and pairwise percent identity calculations. For Figure S1, the alignment was manually edited to span the region covered by pyrotag sequencing, and columns in the alignment with a majority of gaps were removed. *Methanocaldococcus jannaschii* (Genbank: M59126.1) was used as the outgroup to root the trees.

Glycoside Hydrolase Activity Assays

The glycoside hydrolase activities present in each switchgrass-adapted supernatant were measured using the DNS reducing sugar assay (endoglucanase, xylanase) and the *p*-nitrophenol (*p*NP) assay (β-D-glucosidase, cellobiohydrolase, β-D-xylosidase and α-L-arabinofuranosidase) (5, 30). Heat-killed samples generated by heating the supernatant to 95°C for 16 h were used as blanks. Activity units for all assays were calculated as μmol sugar liberated min^{-1} ml^{-1} and reported as U/ml. The endoglucanase and xylanase activities of the JP/Z-9 1%SG, and JP/Z-9 SGCMC supernatants were also measured using zymography with SDS-PAGE gels embedded with CMC and oat spelt xylan. Detailed procedures are described in Supplemental Methods.

Saccharification of IL-Pretreated Switchgrass

The supernatant from the JP-9 1%SG switchgrass-adapted community was tested for its ability to saccharify ionic liquid-pretreated switchgrass. The switchgrass was pretreated by dissolution in [C2mim][OAc] at 140°C for three hours and precipitation with an acetone/ethanol mixture. The precipitated material was successively washed with ethanol

and water to remove residual ionic liquid (8). Duplicate 10 ml saccharification reactions were set up with 250 mg of IL-pretreated switchgrass. The JP-9 1%SG reaction consisted of 9.5 ml of pre-warmed supernatant mixed with 0.5 ml of 1 M NaOAc pH 5.0. For comparison, commercial enzyme preparations containing both cellulase and xylanase activities were mixed using recommended enzyme/glucan content of biomass (w/w) loadings; 8.32 µl of NS50013 (1% w/w) and 0.832 µl each of NS50010 and NS50030 (0.1% w/w) were added to 10 ml pre-warmed 100 mM NaOAc pH 5.0 buffer. Lower amounts of enzymes were used to test thermo- and IL-tolerance due to limited amounts of the JP-9 1%SG supernatant. Lyophilized supernatant (1 ml) was resuspended in 1 ml of 100 mM NaOAc pH 5.0 and added to 9 ml of the same pre-warmed buffer. The Novozymes enzyme preparations were adjusted to match more closely the endoglucanase and xylanase activity of the JP-9 1%SG supernatant: 0.25 µl of the cellulase mix (8.32 µl of NS50013 and 0.832 µl of NS 50010) and 2.5 µl of the xylanase NS50030 were added to 10 ml pre-warmed buffer. For IL-tolerance, the reaction buffer used was 100 mM NaOAc/15% [C2mim][OAc] at pH 5.0. All samples were incubated in a shaker for 72 h at 70°C or 80°C, and 150 µl was withdrawn for each time point and frozen at -20°C. Time point samples were then spun at 21,000 *x g* for 5 min at 4°C and 5 µl was added to 55 µl water and 60 µl DNS reagent. Samples were incubated at 95°C for 5 min, and ABS 540 nm was taken. A background subtraction blank was made by adding 5 µl of each sample to 115 µl of water. The total sugars were calculated by comparing to a standard curve of glucose. The percent total sugar was calculated using the estimated glucan and xylan content of switchgrass (47% and 33%, respectively).

Chemicals

All chemical were reagent grade and were obtained from Sigma (St. Louis, MO) unless otherwise noted and used as received.

Nucleotide Accession Numbers

Reads from 454 pyrosequencing are available in the NCBI Short Read Archive: JP 6%SG (SRX044645), JP 1%SG (SRX044646), JP SGCMC (SRX044647), Z 6%SG (SRX044648), Z 1%SG (SRX044649), Z SGCMC (SRX044650). Illumina sequencing reads used in assembly of 16S rRNA gene sequences are available in the NCBI Short Read Archive: JP 1% SG (SRX040418), Z 1%SG (SRX040352). EMIRGE-derived sequences were deposited in the GenBank database under accession numbers JN091905-JN091925.

Results

Enrichment of Thermophilic Bacterial Consortia on Switchgrass

Enrichment cultures with inocula sourced from two compost facilities in Northern California were adapted to switchgrass (6% w/v) through multiple passages at 60ºC. The compost inocula were complex communities composed of 259 taxa for the Jepson Prairie enrichment (JP) and 593 taxa for the Zamora enrichment (Z). A significant reduction in taxonomic diversity was observed in the initial adaptation to switchgrass at 60℃. This reduction in complexity was demonstrated by a reduction in the Shannon diversity index from 3.87 in the initial JP inoculum to 2.04 in the first JP enrichment and from 4.42 to 2.28 for the first Z enrichment (data not shown). These low diversity communities were maintained through nine passages on switchgrass. Additional cultures were generated at later passages by lowering switchgrass loadings to 1% or by amending 1% switchgrass cultures with 0.5% carboxymethylcellulose (CMC), affording six independent samples for microbial community analysis and glycoside hydrolase measurements (Table 1).

Comparison of SSU rRNA amplicons recovered from the final cultures at the level of individual OTUs (97% identity) demonstrated that the enrichment cultures were dominated by only a few phylotypes (Table 2)(Figure 1A-B)(10). Despite the different inocula from which they were derived, the JP and Z enrichment cultures had remarkably similar community profiles. The most abundant sequences recovered from both the high and low biomass JP and Z enrichments were closely related to thermophilic gram-positive *Firmicutes* of the *Paenibacillaceae* family (*Thermobacillus, Paenibacillus*) and *Rhodothermus marinus*, a known biomass deconstructing member of the *Bacteriodetes*. Sequences related to thermophilic *Chloroflexi* (*Thermobaculum, Sphaerobacter, Thermomicrobium*), were recovered at lower abundances, as were sequences related to an

uncultivated lineage, *Gemm-5*, in the *Gemmatimonadetes* phylum. A significant difference between the JP and Z enrichments was the prominence of bacteria closely related to *Thermus thermophilus* in the JP enrichments that was nearly absent in the Z enrichments. However, in the Z enrichments, sequences related to the family *Truperaceace* in the *Deinoccocus/Thermi* phylum were recovered. When CMC was added as a co-substrate, the proportion of amplicons related to *Rhodothermus marinus* increased in both the JP and Z cultures (Figure 1A-B).

Near full-length SSU rRNA sequences (1200-1600 bp) were reconstructed at 97% identity from metagenomic data obtained for JP-9 1%SG and Z-9 1%SG enrichment cultures. These sequences were reconstructed by the EMIRGE method, which uses an expectation maximization algorithim to reconstruct SSU rRNA gene sequences using Illumina metagenomic sequencing data (26). Comparison of the near full length SSU sequences with a maximum likelihood tree confirmed that the two enrichments contained closely related communities (Figure 2). For most members of the enrichment cultures, the reconstructed full length SSU sequences contained identical ≈200bp segments to a corresponding representative pyrotag cluster sequence, validating the ability of the EMIRGE method to reconstruct SSU genes (Figure S1).

The EMIRGE method also predicts the relative abundances of microbes in mixed consortia by probabilistically measuring the relative proportions of reads recruited to each reconstructed SSU rRNA gene sequence (26). For the JP and Z enrichments, EMIRGE abundance estimates showed good general concordance with abundance estimates made from pyrotag sequencing (Table 2). However, the estimated abundance of the thermophilic *Paenibacilli* was lower for the full length sequences compared to the

pyrotags and for the JP enrichment, the abundance of the sequences related to *Thermus thermophilus* was higher compared to the pyrotags. For both enrichments, the estimated abundance of the *Gemmatimonadetes* was higher than predicted by pyrotag abundance, and *Trueperaceae* were predicted at higher abundance in the Z enrichment by EMIRGE.

Glycoside Hydrolase Activities in Switchgrass-Adapted Cultures

Comparison of endoglucanase and xylanase activity at 70°C and pH 5.0 in supernatants obtained from the JP/Z 6%SG and JP/Z 1%SG cultures indicated that more activity was recovered from the 1% switchgrass cultures, so the activity profiles of these supernatants were studied in detail. The supernatants displayed significant enzymatic activity in standard assays, including endo/exoglucanase, β-glucosidase, endoxylanase, β-xylosidase, and α-L-arabinofuranosidase activities (Figure 3A-B and Figure S2). Endoglucanase and xylanase activities were compared to commercial cellulase and xylanase cocktails produced by Novozymes, diluted to comparable activity levels.

Temperature profiles of endoglucanase activity demonstrate that these supernatants possess more thermostable/active enzymes than the commercial cocktails from Novozymes (Figure 4A). Higher optimum temperatures were observed for the endoglucanases recovered from the 1% switchgrass enrichments compared to Novozymes enzymes preparations (T_{opt}-80 °C vs, 60°C). These supernatants even retained endoglucanase activity (15-50%) at 99ºC. Their endoglucanase enzymes also exhibited high levels of tolerance to the ionic liquid [C2mim][OAc], retaining ~50% activity in the presence of 30% [C2mim][OAc], while the Novozymes endoglucanase cocktail was essentially inactive at 10% [C2mim][OAc] (Figure 4B). In contrast,

xylanase activities of the culture supernatants exhibited only a slightly higher thermotolerance and tolerance to [C2mim][OAc] than the Novozymes xylanase preparation (Figure S3 and Figure S4).

Zymograms with CMC and xylan as substrates were performed on the JP and Z 1%SG supernatants (Figure S5). More than 10 bands were observed for each activity, indicating that the enriched consortia each produced multiple endoglucanase and xylanase enzymes. Comparison of the JP and Z samples indicates that the active enzyme complements in both samples were remarkably similar, suggesting that similar communities secrete the endoglucanase and xylanase enzymes. Increased intensity of the some of the endoglucanase bands was observed in the cultures amended with CMC, consistent with the observed increase in endoglucanase activity (Figure 3).

Glycoside Hydrolase Activities on Ionic Liquid-pretreated Switchgrass

Though the supernatants obtained from the switchgrass-adapted communities performed well on model biomass substrates, a more stringent test of their utility is their ability to efficiently deconstruct pretreated biomass. The supernatant obtained from the JP-9 1% SG enrichment performed well on model substrates and was selected to saccharify switchgrass pretreated with [C2mim][OAc]. For comparison, Novozymes enzyme preparations were mixed at the recommended enzyme/biomass loadings (w/w), which amounts to approximately 15x the endoglucanase and 0.25x the xylanase activity compared to the JP-9 1%SG supernatant at 70°C. Despite the comparatively low endoglucanase activity in the JP-9 1%SG supernatant, both cocktails liberated virtually

all the sugars from the [C2mim][OAc]-pretreated switchgrass at 70°C after 72 h (Figure 5A).

The enzyme cocktails were then compared at a higher temperature (80°C) or in 15% [C2mim][OAc] at 70°C (Figure 5B-C). Due to limited amounts of sample, the JP-9 1% SG supernatant was diluted ten-fold in these saccharifications. The Novozymes cocktail enzyme loads were adjusted to approximate the activity levels of the JP-9 1% SG supernatant enzymes using endoglucanase and xylanase assays. At these enzyme loadings only about 40 to 50% of the total sugars were liberated from [C2mim][OAc]-pretreated switchgrass at 70°C. At 80°C, the Novozymes cocktail liberated 73% less sugars in 72 h than at 70°C, while the JP-9 1% SG supernatant liberated about 31% less sugars. In 15% IL, the Novozymes cocktail also liberated fewer sugars after 72 h (81% less than in 0% IL), while the JP-9 1% SG supernatant liberated only 29% less sugars. At 80°C and at 70°C in the presence of 15% [C2mim][OAc], the sugar release profile suggests that the Novozymes cocktail was inactivated within the first 2 h, while the JP-9 1% SG supernatant was active throughout the 72 h incubation.

Discussion

We found that growing compost microbial communities on extracted switchgrass under thermophilic conditions generated simplified bacterial consortia that produced glycoside hydrolase enzymes that are more stable than commercial fungal cocktails when assayed on pretreated switchgrass at high temperature and in the presence of pretreatment chemicals. Adaptive cultivation on other feedstocks such as lignin, cellulose and CMC has also generated simplified communities and, along with this study, indicate that this method is a useful tool for developing simplified biomass-degrading consortia tailored to deconstruct a designated feedstock under defined conditions such as temperature or pH (6, 23, 27).

Both the JP and Z enrichments converged to closely related consortia with similar microbial community compositions and levels of secreted glycoside hydrolase activities. Perturbation of the enriched communities by inoculation into cultures with low biomass (1% switchgrass) and amendment with CMC did not significantly alter the community composition of the enrichments, although the glycoside hydrolase activities measured in the supernatant were higher. This observation indicates that biomass loading is a critical component of enrichment cultivation studies and can be adjusted to maximize titers of enzymes of interest without dramatically affecting the community composition.

The success of this enrichment strategy was demonstrated by the selection of biomass-deconstructing bacteria. Analysis of amplicon pyrosequencing data and reconstructed SSU rRNA genes from metagenomic sequencing demonstrated that thermophilic *Paenibacilli* and *Rhodothermus* groups were abundant in both enrichments. Both of these groups have cultured relatives known to degrade biomass, suggesting that

they are the source of many of the glycoside hydrolases present in the culture supernatants (2, 32, 36). Additionally, compost and swine waste-derived microbial communities enriched on microcrystalline cellulose at elevated temperatures under aerobic conditions also contained thermophilic *Paenibacilli*, suggesting that these microbes are critical components of biomass deconstruction under thermophilic aerobic conditions (23, 27).

Bacteria closely related to *Thermus thermophilus* were dominant members of the JP enrichment but were absent in the Z enrichment. This observation suggests that the *T. thermophilus* may not be involved in biomass deconstruction as the glycoside hydrolase activities and the CMC/xylan zymography are very similar in both enrichments. However, *T. thermophilus* strains have been isolated from hot composts (60-80°C) and shown to express highly active xylanases (15). Detailed proteomic analysis of the secreted proteins in the supernatant and isolation of *T. thermophilus* strains from the enrichment cultures will resolve its role.

Members of the *Chloroflexi* phylum are ubiquitous in the enrichments, and are of interest as the *Chloroflexi* have relatively few cultivated and sequenced representatives, (7, 16, 17, 37). Inspection of genomes available for three thermophilic type strains belonging to this phylum (*T. terrenum*, *Sphaerobacter thermophilus*, *Thermomicrobium roseum*), indicates that they possess a number of cellulases and hemicellulases (21). Another prominent group present in both the JP and Z enrichments is a member of an uncultivated lineage, (*Gemm-5*), in the *Gemmatimonadetes* phylum (38), that is distantly related to the sole cultivated representative of this phylum, *Gemmatimonas aurantica* (SSU rRNA genes have 88% identity). Since both the *Chloroflexi* and

Gemmatimonadetes are commonly found in soils and sediments, sequencing these consortia will illuminate how they function in biomass deconstruction.

A significant amount of xylanase activity was recovered from supernatants isolated from the switchgrass-adapted consortia. The xylanase activity is comparable to highly productive *Bacillus* and *Paenibacillus* strains cultivated under optimized conditions, suggesting that mixed consortial cultivation on switchgrass is an effective method to generate high levels of xylanase enzymes (19). Celluloytic activity was relatively low and cultivations on pretreated switchgrass are being explored to enhance the cellulolytic activity of the recovered supernatants. Comparison of the endoglucanase activity between the culture supernatants and the Novozymes preparations suggest that the thermophilic bacteria secrete enzymes that are both more thermotolerant and more active in the presence of ionic liquid [C2mim][OAc], a property that has been observed for purified enzymes (5). This correlation will be confirmed by purifying endoglucanases from the supernatant and performing assays on the purified proteins in the presence of [C2mim][OAc]. Interestingly, the thermotolerance and the [C2mim][OAc]-tolerance of the xylanase activities of the culture supernatants and the Novozymes preparations were comparable, suggesting the properties of the xylanases in the culture supernatants and the Novozymes xylanase preparation (NS50030) may be similar. Zymography revealed that the thermophilic consortia produce multiple enzymes for at least two of the glycoside hydrolase activities profiled. These enzymes are currently being identified by a combination of metagenomic sequencing and mass-spectrometry-based proteomic measurements.

Both the Novozymes enzyme preparations and the culture supernatants were able to saccharify [C2mim][OAc]-pretreated switchgrass at 70°C. These observations indicate that both systems possess the necessary complement of glycoside hydrolase enzymes to release sugars from complex biomass. Surprisingly, the Novozymes preparations efficiently released sugars from pretreated switchgrass at 70°C despite that temperature being 10°C above the cellulase optimum temperature and 20°C higher than the recommended reaction temperature (14). Perhaps this is due to the greatly enhanced hydrolysis kinetics of [C2mim][OAc]-pretreated switchgrass compared to acid-pretetreated switchgrass, allowing for rapid polysaccharide hydrolysis before enzyme denaturation (22). However, at 80°C or in the presence of ionic liquids, the enhanced stability of the glycoside hydrolase enzymes from the thermophilic bacterial cultures is evident (Figure 5B-C), demonstrating their value as sources of enzymes for enzymatic cocktails adapted to more extreme reaction conditions than fungal enzymes can tolerate.

In conclusion, this study demonstrates that thermophilic, aerobic microbial consortia cultivated from compost produce significant titers of glycoside hydrolase enzymes that can be directly compared to commercial fungal biomass-deconstructing cocktails. Microbial community analysis has demonstrated that these consortia are composed of a few dominant phylotypes that consist of both well-studied and novel biomass-deconstructing bacteria. Therefore, these consortia are amenable to detailed genomic and proteomic investigations which will reveal the suite of bacterial glycoside hydrolases used to deconstruct complex biomass (34). This approach will allow characterization of new bacterial glycoside hydrolases and accessory proteins from uncultivated organisms that will enhance biomass deconstruction.

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Table 1: Switchgrass-adapted Communities

Culture ID[1]	Feedstock (% w/v)
JP-9 6%SG	Switchgrass (6%)
JP-9 1%SG	Switchgrass (1%)[2]
JP-9 SGCMC	Switchgrass (1%), Carboxymethylcellulose (0.5%)[3]
Z-9 6%SG	Switchgrass (6%)
Z-9 1% SG	Switchgrass (1%)[2]
Z -9 SGCMC	Switchgrass (1%), Carboxymethylcellulose (0.5%)[3]

[1]The Culture ID represents the final culture used for analysis of microbial community composition and glycoside hydrolase activties.

[2]Enrichments with 1% switchgrass were inoculated with 6% enrichment after the seventh two week passage of the enrichment.

[3]Enrichment with 1% switchgrass/0.5% switchgrass were inoculated with the 6% enrichment after the eighth passage of the enrichment.

Table 2. Comparison of Estimated Abundances for Pyrotag and Metagenomic Sequencing for JP-9 1%SG and Z-9 1%SG[1]

Groups	JP- 9 1%SG		Z-9 1%SG	
	Pyrotags	EMIRGE	Pyrotags	EMIRGE
Thermophilic *Paenibacilli*[2]	40.4	20.4	69.7	49.9
Thermus thermophilus	39.3	49.0	<1.00	<1.00
Rhodothermus marinus	9.62	10.4	17.2	18.4
Gemm-5[3]	2.05	7.70	3.15	13.5
Thermomicrobia	1.18	<1.00	2.41	1.83
Thermobaculum terrenum	1.43	1.40	<1.00	<1.00
Sphaerobacter thermophilus	<1.00	1.16	<1.00	<1.00
Trueperacae	<1.00	<1.00	1.01	7.88

[1]Groups with >1% estimated abundance in pyrotag or EMIRGE-derived sequences are depicted in the table. Total estimated abundances of these depicted groups are 90-95% of the total community.

[2]Pyrotags abundances are represented by sum of the abundances for pyrotag clusters related to *Paenbacillus* sp. str. SAFN-007, *Paenibacillus kobensis* DSM 10249, *Paenibacillus* D273a and *Thermobacillus* sp. str. KWC4. EMIRGE-derived sequence abundance is the sum of individual sequences presented in Figure 3 that cluster with *Paenibacillacae* family (JP 211, JP 261, JP 2339, JP 2453; JP 2459, Z 19, Z51, Z 137, Z 146, Z 261, Z 484, Z 1266, Z 1300). The individual estimated abundances of each of these sequences are listed on the phylogenetic tree in Figure S1.

[3] *Gemm-5* is *Gemmontimonadetes* subdivision 5.

Figure Legend

Figure 1. Plot of the relative abundance of dominant taxa (≥1%) based on SSU rRNA amplicon pyrosequencing for the switchgrass-adapted enrichments: (A) Jepson Prairie enrichments (JP 6%SG, JP 1%SG, JP SGCMC); (B) Zamora enrichments (Z 6%SG, Z 1%SG, Z SGCMC). A cutoff of 1% abundance was chosen to highlight the most abundant organisms present in the community. OTUs that were assigned the same name by Pyrotagger are distinguished by adding the pyrotagger cluster number after the name. GenBank accessions numbers for each cluster assigned by Pyrotagger are included in Supplemental Table 1A (JP) and 1B (Z). Amplicon library size: JP 6%SG (2004), JP 1%SG (2107), JP SGCMC (1496), Z 6%SG (3095), Z 1%SG (3095), Z SGCMC (2310).

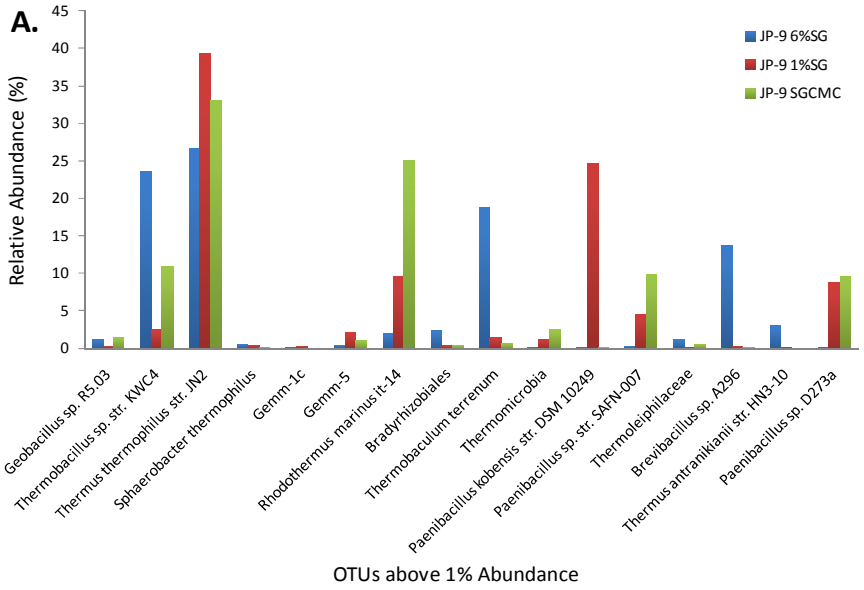
Figure 2. Maximum likelihood phylogenetic tree of 1% SG enrichment communities. Full-length SSU sequences reconstructed with EMIRGE are shown for both Z (circles) and JP (diamonds) communities, along with selected reference sequences identified from pyrotag sequencing of the initial inocula, the first switchgrass enrichment and the ninth enrichment (Table S1A-1B). Bootstrap support values >50% are shown at the nodes on the tree. Units are base substitutions per site. EMIRGE-derived sequences are available as Supplemental File for this manuscript.

Figure 3. (A) endoglucanase and (B) endoxylanase activities measured in the supernatant of the final switchgrass-adapted cultures at 70°C and pH 5.0. Novozymes cellulase and xylanase cocktails were used as positive controls. The Novozymes NS5003-10 and NS50030 enzymes were diluted 1:1000 and 1:10000, respectively, concentrations that are similar to those used to saccharify biomass at 2.5% w/v loadings in Figure 5A. Endoxylanase values reported were extrapolated from 1:10 dilutions of supernatant sample.

Figure 4. (A) Temperature profiles and (B) ionic liquid tolerance profiles of the endoglucanase activities of the low biomass and CMC amended switchgrass-adapted community culture supernatants. The Novozymes cellulase positive control is a mix of NS50013 and NS50010 enzyme products. For the profile of ionic liquid tolerance, the reactions were incubated at 50℃ and pH 5.0.

Figure 5. Saccharification of ionic liquid-pretreated switchgrass incubated with either the JP-9 1%SG supernatant or a Novozymes cellulase (NS50013-10)/xylanase (NS50030) cocktail. Saccharification using (A) undiluted supernatant and the Novozymes cocktail mixed at recommended enzyme loadings, incubated at 70℃ pH 5.0 or (B-C) 1:10 diluted supernatant and the Novozymes cocktail mixed with endoglucanase and xylanse enzyme activity levels matching the supernatant, incubated at 70℃ and either (B) 80℃ or (C) with 15% of [C2mim][OAc] at 70℃, each reaction at pH 5.0.

Figure 1



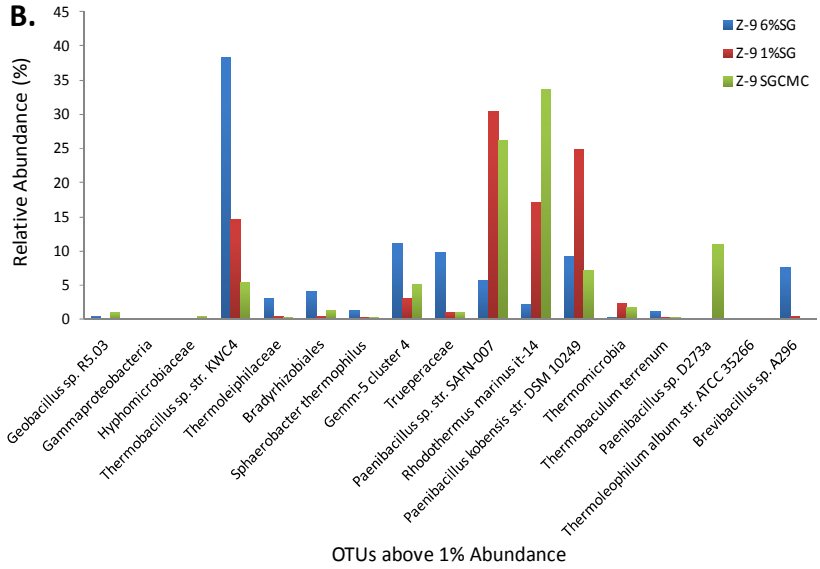


Figure 2

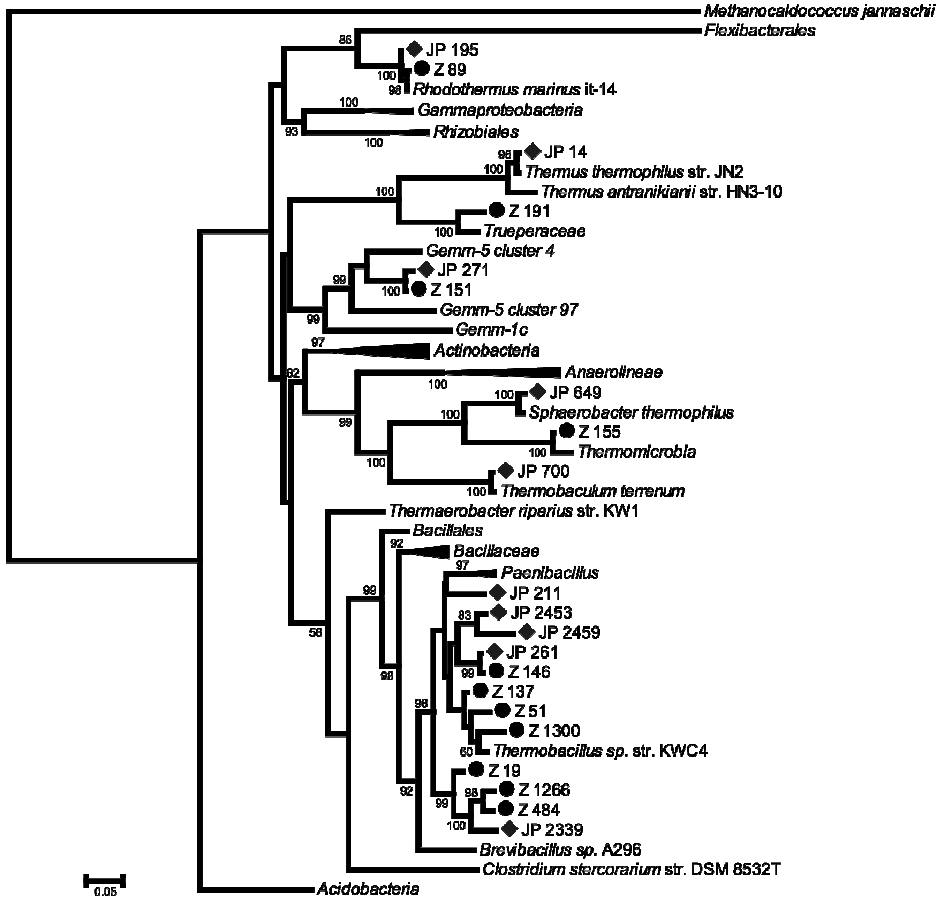


Figure 3

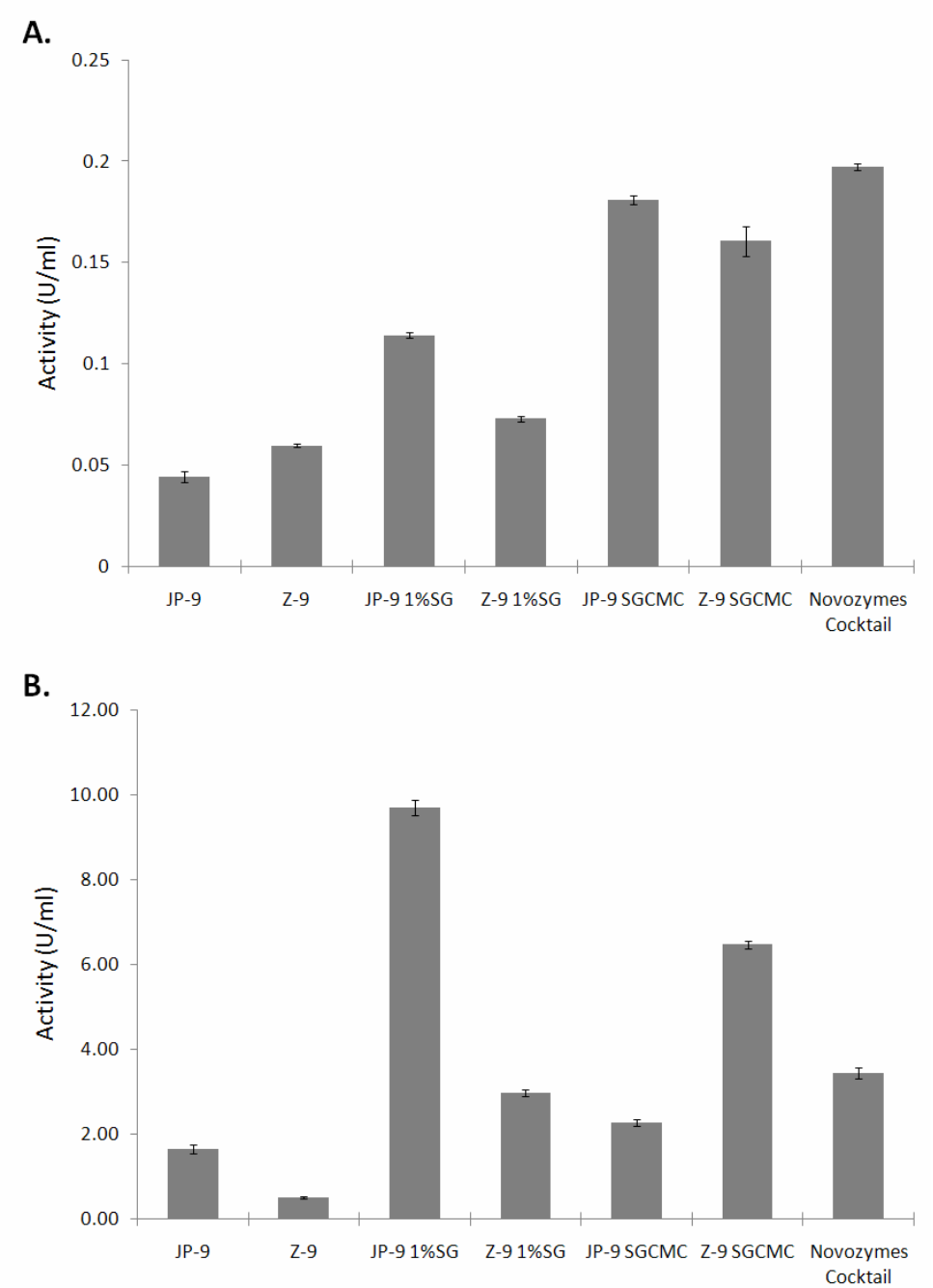
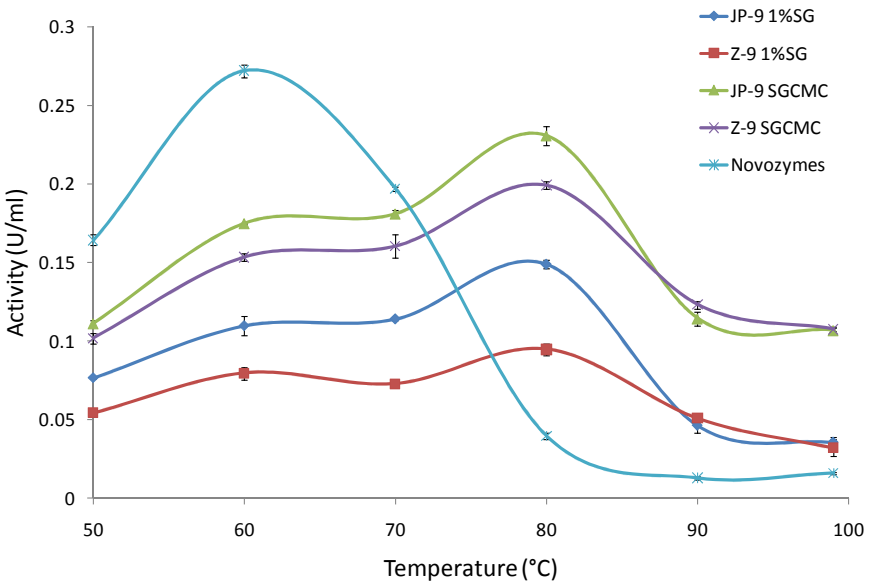


Figure 4

A



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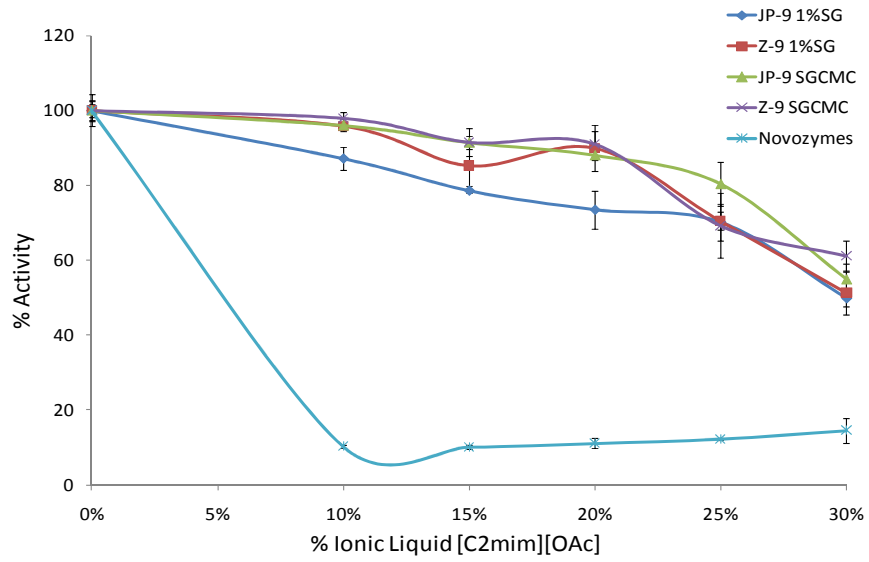
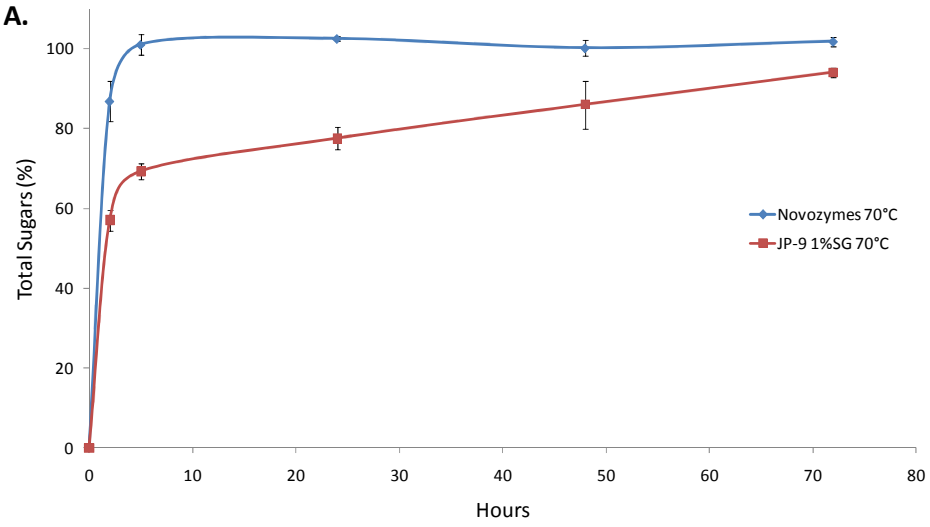
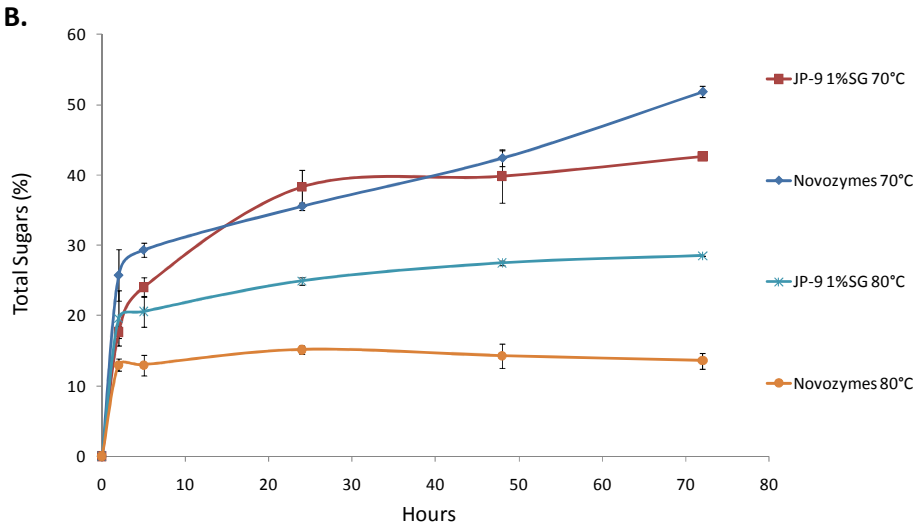
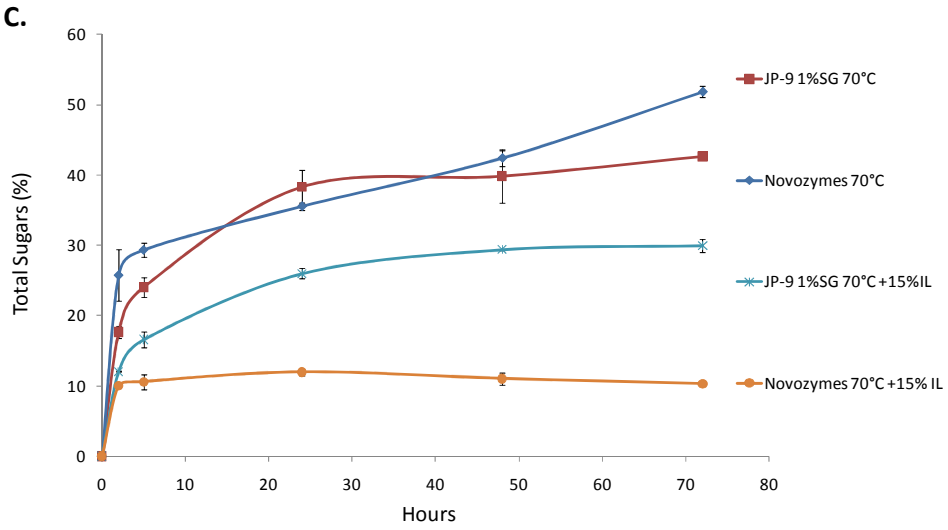


Figure 5





Thermophilic Switchgrass-Adapted Consortia



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